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Influence of Cathepsin D Expression in Lung Adenocarcinoma on Prognosis: Possible Importance of Its Expression in Tumor Cells and Stromal Cells, and Its Intracellular Polarization in Tumor Cells

MASAHIKO HIGASHIYAMA, MD,[1]* OSAMU DOI, MD,[1] KEN KODAMA, MD,[1]
HIDEOKI YOKOUCHI, MD,[1] TSUTOMU KASUGAI, MD,[2] AND SHINGO ISHIGURO, MD[2]
[1]*Department of Thoracic Surgery, Osaka Medical Center for Cancer and Cardiovascular Diseases, Osaka, Japan*
[2]*Department of Pathology, Osaka Medical Center for Cancer and Cardiovascular Diseases, Osaka, Japan*

Background: Cathepsin D, an aspartic lysosomal proteinase, has been described to be closely associated with tumor progression and prognosis in some human malignancies. The purpose of this study was to determine clinicopathological and prognostic significance of cathepsin D expression in lung adenocarcinoma.

Methods: Expression of cathepsin D in 152 lung adenocarcinoma patients was immunohistochemically studied using the antihuman cathepsin D antibody.

Results: Eighty patients (53%) showed negative immunoreactivities in tumor cells. The cathepsin D-positive patients (72 patients, 47%) were divided into two subgroups; granular type expression (48 patients, 31%) with its polarized expression mainly in the luminal side of the cytoplasm of tumor cells and basal type expression (24 patients, 16%) with its polarized expression mainly in the basal or infranuclear side of the cytoplasm. Patients with basal type expression showed significantly more marked scar formation ($P = 0.042$), and especially among the patients with stage I disease, those with basal type tended to show poorer prognosis ($P = 0.071$) than the others. Cathepsin D was also expressed in stromal cells within the tumor tissues, and 86 patients (57%) with moderate to massively infiltrating cathepsin D-positive stromal cells showed a lower grade of differentiation ($P = 0.005$) and higher scar grade ($P = 0.0003$) than those with few cathepsin D-positive stromal cells. Cathepsin D status in stromal cells was significantly associated with prognosis ($P = 0.014$), and in a multivariate analysis, its expression status in stomal cells was marginally an independent prognostic factor only among the stage I patients.

Conclusions: In determining significance of cathepsin D expression in this disease, it is important to consider separately its expression cell type and its polarization pattern in tumor cells within the tumor tissue. How-

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*Correspondence to: Masahiko Higashiyama, M.D., Department of Thoracic Surgery, Osaka Medical Center for Cancer and Cardiovascular Diseases, Nakamichi 1-3-3, Higashinariku, Osaka, 537, Japan
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ever, only cathepsin D status in stromal cells within the tumor tissue is a marginal marker influencing prognosis among stage I patients.

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KEY WORDS: cathepsin D; lung adenocarcinoma; prognosis; immunohistochemistry; tumor cell; stromal cell

INTRODUCTION

Recent studies [1–4] have suggested that cathepsin D, an aspartic lysosomal proteinase, possesses proteolytic function for proteoglycan substrates, such as stromal matrix and basement membrane, as well as promoting function for cell proliferation. Considering these biophysiological functions of this proteinase, we have been motivated to clarify its significance in tumor tissues. In fact, many investigators have studied the expression of cathepsin D in various types of tumor tissue [5–7]. In particular, in breast cancer, several studies showed that this proteinase may be closely associated with tumor progression and prognosis [8–14]. However, some investigators [15,16] found no association between this proteinase expression and tumor malignant potency, and Henry et al. [17] showed its inverse association. Thus, although these conflicting results may relate to differences, not only in patient populations but also in methodology, its significance even in breast cancer is now controversial [7].

In contrast to breast cancer, few studies have been reported in other types of tumors. In non-small cell lung cancer, to our knowledge, only a few reports describe cathepsin D expression [18,19], but the materials in the reports included various histological types and the number of the examined tumors was relatively small. Thus, the purpose of this study was to determine clinicopathological and prognostic significance of cathepsin D expression in the tumor tissue of patients with lung adenocarcinoma.

MATERIALS AND METHODS

Patients

The current study included 152 lung adenocarcinoma patients, who underwent potentially curative operation in our institute between 1983 and 1989. The patients comprised 99 men and 53 women, with ages ranging from 19 to 79 years (mean 61.8) and with a median follow-up of 52.5 months (range 8.8–125.7).

Clinicopathological Status

The stages of the subjects in this study were classified according to the international classification of the pathological TNM staging system [20]; 74 patients were pathological stage I (p-stage I), 16 p-stage II, and 62 p-stage IIIA. Histological differentiation were based on the classification of the Japan Lung Cancer Society [21]. Scar formation was categorized into four grades, as described by Shimosato et al. [22]: No and minor desmoplasia was regarded as grades I and II, respectively, an abundant amount of collagen formation as grade III, and hyalinized tissue as grade IV.

Immunohistochemical Assay

Immunohistochemical staining was performed as described elsewhere [14–18]. Briefly, 4-μm sections from routinely formalin-fixed and paraffin-embedded blocks, including the maximum cut-surface of each tumor tissue, were deparaffinized in xylene, rehydrated, and incubated for 15 min with 3% hydrogen peroxide in methanol. After pretreatment with normal goat serum, rabbit anticathepsin D polyclonal antibody diluted 1 : 200 (Novocastra, Newcastle, UK) was added and incubated for 60 min [15,17]. Tests by the manufacturer demonstrated that this antibody was specific for human total cathepsin D. After the second reaction with biotinylated goat antirabbit γ-globulin, and subsequently avidin-biotin peroxidase complex solution, the reactions were visualized with diaminobenzidine (DAB), containing 0.01% hydrogen peroxide. Sections of human liver tissue served as positive controls and, by substituting normal rabbit serum for the primary antibody, as negative controls.

Cathepsin D expression in lung adenocarcinoma tissues was separately assessed on two cell types (i.e., tumor cells and infiltrating stromal cells in the tissues). Patients were grouped into cathepsin D-positives, who showed positive immunostaining in more than 5% tumor cells, and cathepsin D-negatives, who showed no immunostaining or were positive in less than 5% of tumor cells. In addition, cathepsin D-positive patients were divided into two types (i.e., granular type and basal type) (Fig. 1A–D). The former showed mainly granular, often patchy, staining pattern mainly in the luminal area of the cytoplasm, these immunostaining patterns being similar to that in the normal bronchial epithelim (Fig. 1A,B). By contrast, the localization of cathepsin D expression mainly in the basal or infranuclear area of the cytoplasm was a distinctive feature of the latter, which, moreover, often showed a linear staining pattern within the basal portion of the cytoplasmic membrane (Fig. 1C,D). When both patterns were observed within the tumor tissues, the predominant pattern was chosen. On the other hand, the

degrees of cathepsin D-positive stromal cells, such as fibroblast-like cells or macrophages, were semiquantitatively classified into two groups; those with few and those with moderate-to-massive cathepsin D-positive stromal cells infiltrating within the tumor tissues (Fig. 1E). Alveolar macrophage, which often infiltrated into the alveolar spaces or some tubular spaces of tumor cells, also showed strong immunoreactivity for cathepsin D, but in the present study, the degree of cathepsin D-positive cells was analyzed mainly on infiltrating fibroblastic-like cells into the stroma.

Statistical Methods

The chi-square test was used for statistical analysis. The Kaplan-Meier method was used to calculate the postoperative survival rate, and prognostic significance was evaluated by the log-rank test. Differences with $P < 0.05$ were considered significant, and $0.05 \leqslant P < 0.1$ was considered marginally significant. Factors related to survival were analyzed by Cox's proportional hazards regression model [23] with SAS software (Statistical Analysis System Institute, Cary, NC) [23].

RESULTS

Cathepsin D Expression in Tumor Cells

Of the tested 152 samples, while 80 (53%) patients were included in cathepsin D-negatives, cathepsin D-positive tumor cells were detected in 72 (47%) patients within the tumor tissues. These cathepsin D-positive patients were divided into two subgroups, 48 (31%) patients with granular type and 24 patients (16%) with basal type.

No significant correlation of cathepsin D expression in tumor cells was found in p-stage, tumor size, T-factor, nodal involvement, subtype, and differentiation. However, patients with basal type expression revealed a higher degree of scar grade (grade III + IV) than those in the other (sub)groups ($P = 0.042$) (Table I).

Cathepsin D Expression in Stromal Cells

Of 152 patients, 66 (43%) patients showed few cathepsin D-positive stromal cells within the tumor tissues, while the rest (57%) showed moderate to massive cathepsin D-positive stromal cells within the tumor tissues. Table II shows an association between cathepsin D in stromal cells and clinicopathological factors. Bronchioloalveolar type of lung adenocarcinoma significantly showed less cathepsin D-positive stromal cells ($P = 0.015$), and histological differential also correlated with the degree of cathepsin D-positive stromal cells infiltration ($P = 0.005$): patients with less differentiation showed more infiltration of cathepsin D-positive stromal cells. The degree of cathepsin D-positive stromal cells was also significantly correlated with scar grade ($P = 0.0003$).

Association Between Cathepsin D in Tumor Cells and Cathepsin D in Stromal Cells

Table III shows an association between cathepsin D in tumor cells and that in stromal cells. Patients with cathepsin D-positivity in tumor cells showed a significantly higher degree of cathepsin D-positive stromal cell infiltration ($P = 0.0008$). Especially in 24 patients with basal type expression, cathepsin D-positive tumor cells were strongly distributed in the central or fibrous scar area, admixed with the more massive cathepsin D-positive stromal cells, within the tumor tissues (Fig. 1C).

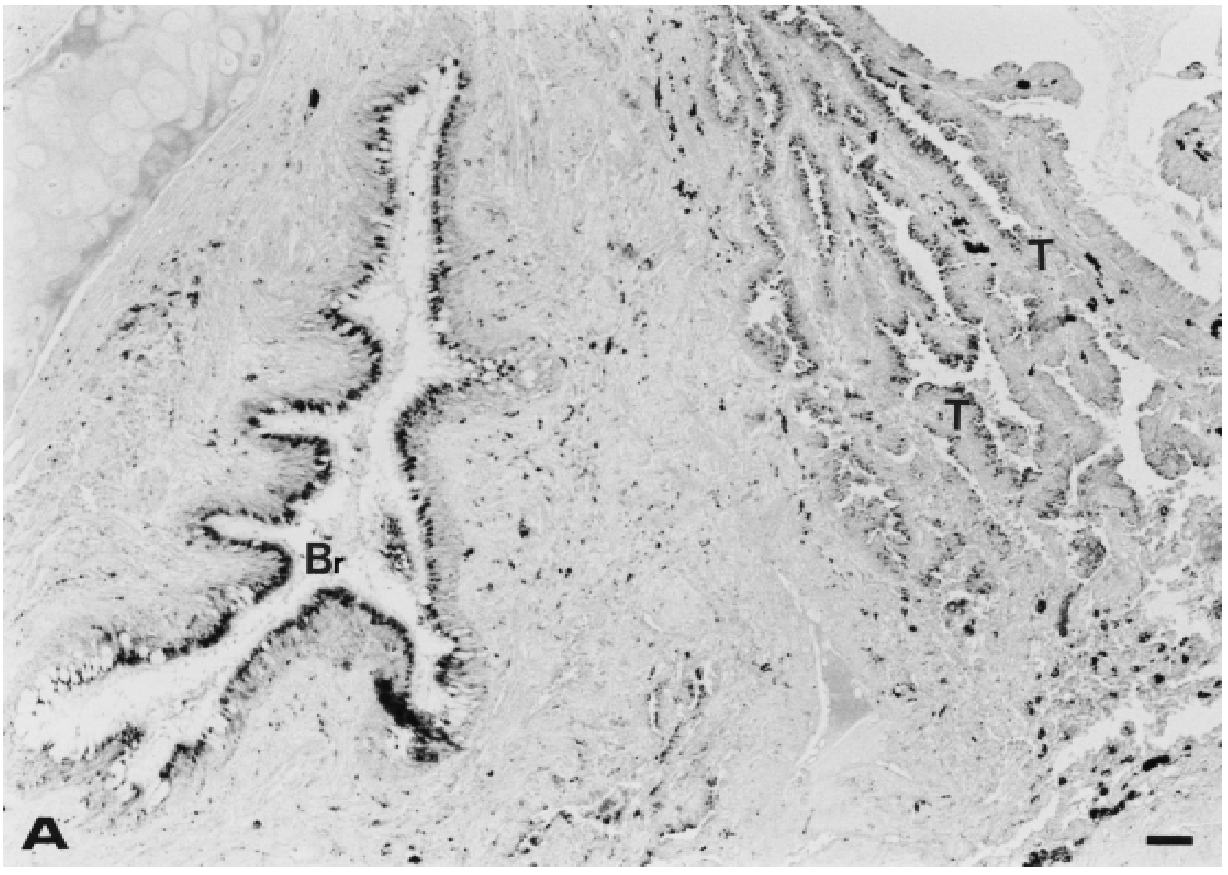
Prognostic Significance of Cathepsin D Expression in Tumor Cells and in Stromal Cells

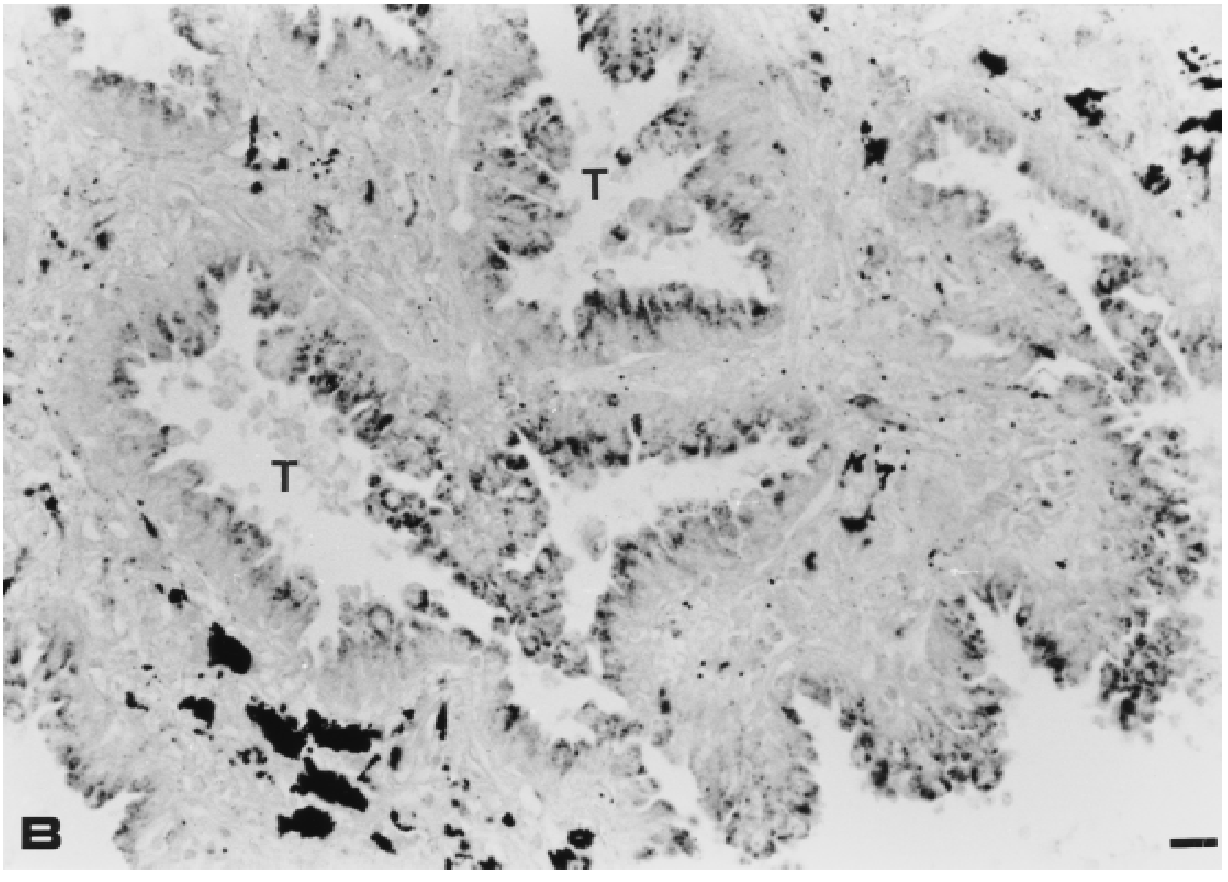
Figure 2A shows the survival curves of the three subgroups with all patients in the present series, and there was no significant difference among the subgroups ($P = 0.306$). As shown in Figure 2B, which included only stage I patients, no overall prognostic value was demonstrated ($P = 0.154$) but, in an individual analysis, patients with basal type expression carried a marginally poor prognosis, compared to those in the other subgroups ($P = 0.071$). By contrast, patients with moderate to massive infiltration of cathepsin D-positive stromal cells carried a significantly poorer prognosis than that of patients with infiltration of few cathepsin D-positive stromal cells (Fig. 2C, $P = 0.014$) and, among stage I patients (Fig. 2D), the same result was observed ($P = 0.029$).

Table IV shows the results of multivariate analyses using variables univariately associated with prognosis. p-stage status was an independent prognostic factor in all patients ($P = 0.006$). However, among stage I patients, cathepsin D status in stromal cells was a marginal prognostic factor ($P = 0.082$), next to tumor size status ($P = 0.035$).

DISCUSSION

In breast cancer [8–16], although cathepsin D expression status has been most widely examined, its clinicopathological and prognostic significance has remained controversial [7]. In general, several analyses of cathepsin D concentrations in cytosolic extracts within the tissues showed a higher total cathepsin D amount to be correlated with more aggressive and less favorable prognosis [8–13], and a biochemical assay using the enzymatic activity of cathepsin D within the tumor tissues demonstrated similar results [12]. By contrast, investigations using immunohistochemical assays [14–17] have not revealed a consistent association between cathepsin





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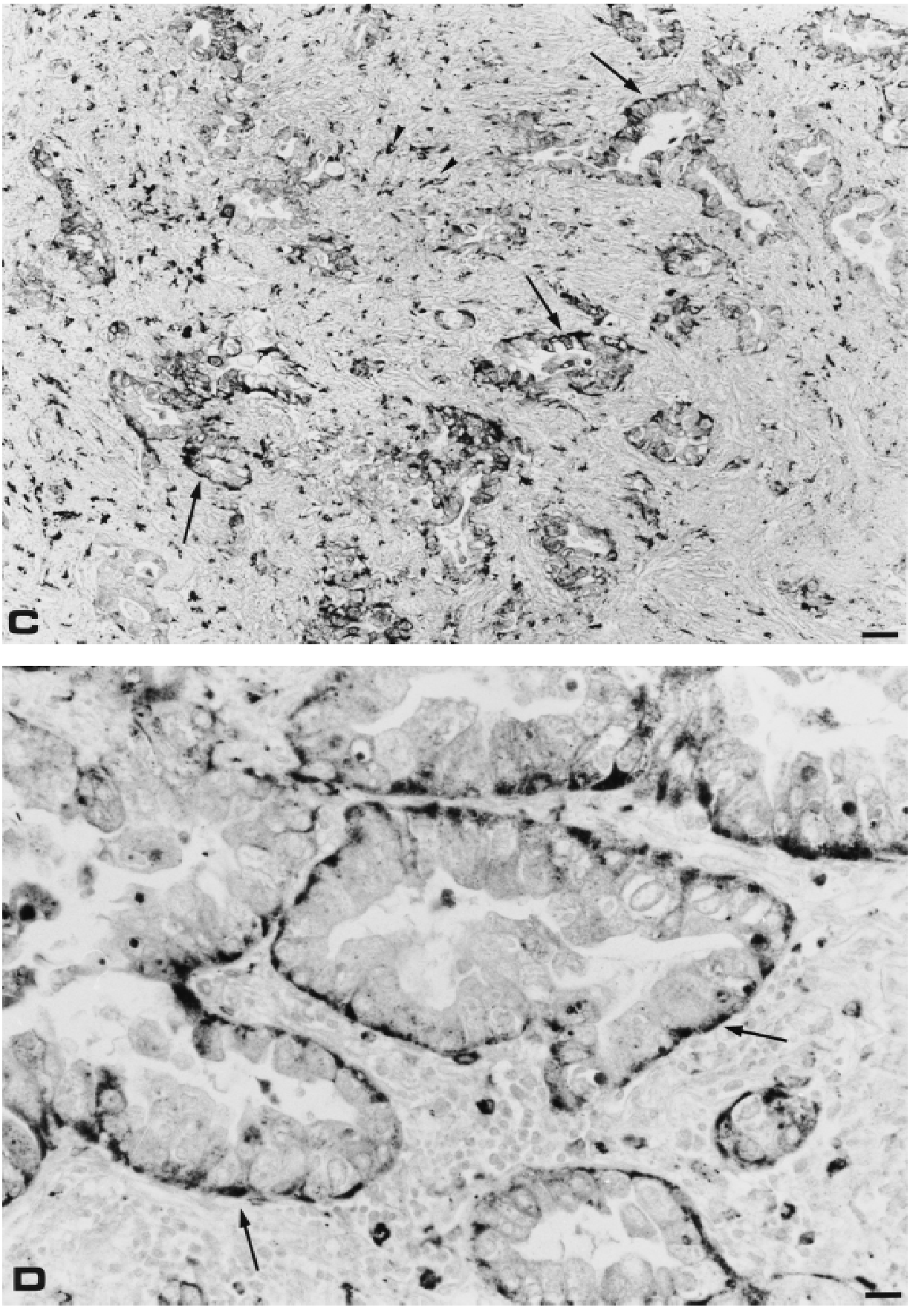

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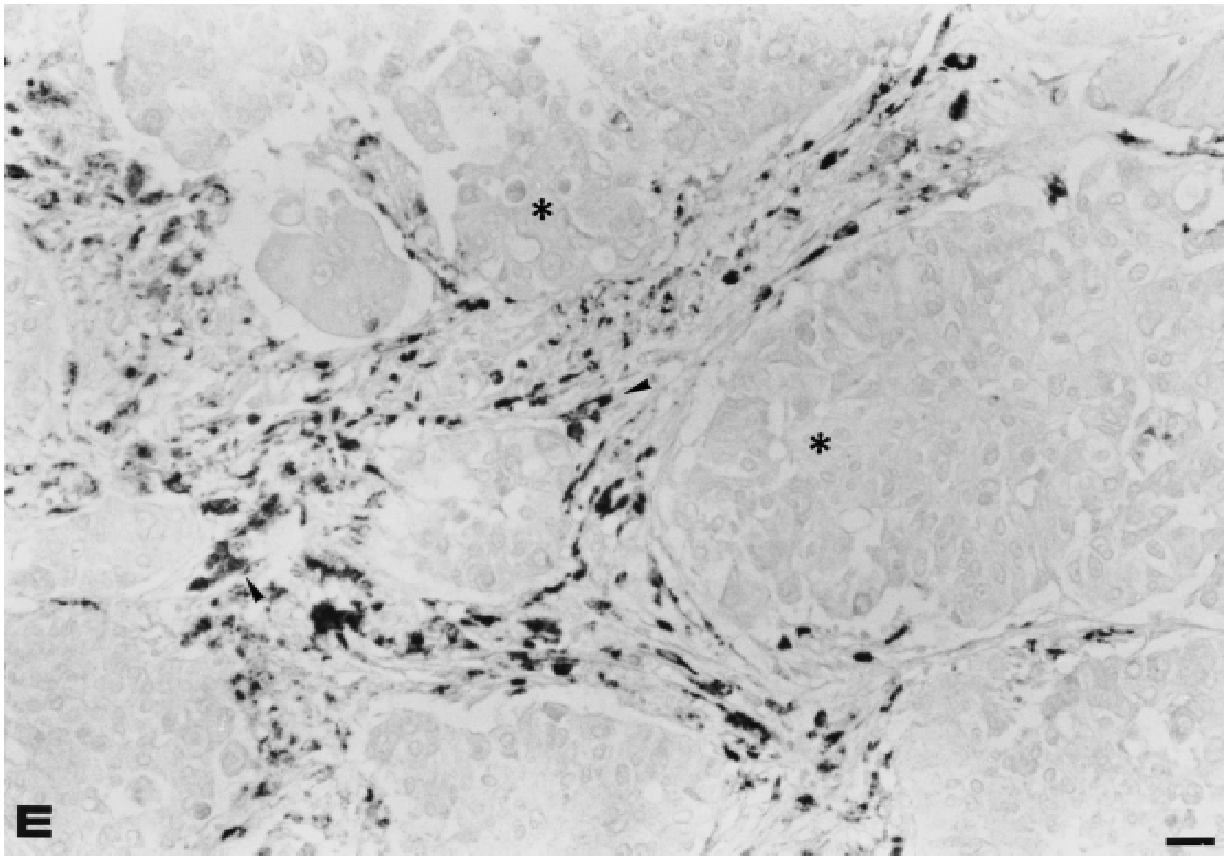

Fig. 1. Immunostaining of cathepsin D in the tissues of lung adenocarcinoma. **A,B:** Granular type. Patients with this type showed granular or patchy staining pattern of cathepsin D mainly in the luminal area of the tumor cell cytoplasm (T). This staining pattern was similarly observed in the normal bronchial epithelia (Br). Bars: **A** = 49 μm; **B** = 15 μm. **C,D:** Basal type. Patients with this type showed granular or linear staining pattern of cathespin D mainly in the infranuclear or basal area of the tumor cell cytoplasm or cytoplasmic membrane (arrow). Bars: **C** = 49 μm, **D** = 19 μm. Stromal cells, such as fibroblastic-like cells or macrophages especially infiltrating within the fibrous scar, also expressed strongly cathepsin D (arrowhead). **E:** Massively infiltrating stromal cells were strongly positive for cathepsin D expression (arrowhead), while none of lung adenocarcinoma cells expressed cathepsin D (*). Bar = 24 μm.

D expression and prognosis. When only the cathepsin D status in tumor cells was immunohistochemically evaluated, its status correlated with the conflicting prognosis. Formerly, Isola et al. [14] emphasized that cathepsin D expression in tumor cells of node-negative breast cancer was an independent prognostic factor influencing diminished survival, while Henry et al. [17] described that its expression, strongly related to estrogen-receptor expression, showed better prognosis and better differentiation, especially among node-positive breast cancer patients. However, in several recent reports [15,16], cathepsin D expression in tumor cells failed to correlate with prognosis. Thus, a difference in methodology to evaluate cathepsin D expression in tumor cells might provide conflicting conclusions.

In lung cancer, Fontanini et al. [18] first detected cathepsin D in non-small cell lung cancer using an immunohistochemical technique, reporting that its expression in tumor cells correlated with a better differentiation, the earlier stage, the better prognosis, but they examined only tumor cells, and the number of adenocarcinoma type was too small (N = 39), suggesting that their analysis may be insufficient to conclude its significance. Recently, Ledakis et al. [19] described no significance of cathepsin D concentration in lung cancer tissue, but their results were based on the total amount of cathepsin D within the tissue.

According to intracellular polarization pattern of cathepsin D expression in tumor cells, we first found two types (i.e., granular type or basal type), in the patients with lung adenocarcinoma. Among stage I patients, these two types showed slightly opposing prognostic significance: Patients with basal type tended to have a poor prognosis ($P = 0.071$), compared to those with granular type and cathepsin D-negative patients. If these two subtypes are mixed together as cathepsin D-positive, the postoperative overall survival curves of cathepsin D-positive and cathepsin D-negative patients show a similar pattern, no prognostic significance being observed even among stage I patients (data not shown).

The staining pattern of granular type was similar to that in the normal bronchial epithelium. This intracellular

TABLE I. Association Between Cathepsin D in Tumor Cells and Clinicopathological Factors

	Negative n = 80 (53%)	Positive: Granular type n = 48 (31%)	Positive: Basal type n = 24 (16%)	*P*
Sex (male/female)	50/30	33/15	16/8	NS
Age (mean)	61.7	59.3	62.2	NS
p-stage				
I	41	20	18	
II, IIIA	39	28	11	NS
Tumor size (mm)				
<31	41	30	10	
31≤ <61	33	16	10	
61≤	6	2	4	NS
T-factor[a]				
T1	36	29	10	
T2	36	17	12	
T3	8	2	2	NS
Nodal involvement				
Negative	48	23	14	
Positive	32	25	10	NS
Subtype[b]				
Tubular	18	16	7	
Papillary	57	32	16	
Br-alv[c]	5	0	1	NS
Differentiation[b]				
Well	31	11	8	
Moderate	30	25	12	
Poor	19	12	4	NS
Scar grade[d]				
I, II	41	23	6	
III, IV	39	25	18	0.042[e]

[a]TNM staging according to Mountain's classification [20].
[b]Histological classification according to the Japanese Lung Cancer Society [21].
[c]Bronchioloalveolar type.
[d]Scar grade by Shimosato et al. [22].
[e]Basal type versus others.
NS, not significant.

TABLE II. Association Between Cathepsin D in Stromal Cells and Clinicopathological Factors

	Grade of cathepsin D-positive stromal cell infiltration: Few 66 (43%)	Moderate to massive 86 (57%)	*P*
Sex (male/female)	35/31	64/22	0.010
Age (mean)	60.4	62.3	NS
p-stage			
I	37	37	
II, IIIA	29	49	NS
Tumor size (mm)			
<31	34	47	
31≤ <61	28	31	
61≤	4	8	NS
T-factor[a]			
T1	31	44	
T2	30	35	
T3	5	7	NS
Nodal involvement			
Negative	41	44	
Positive	25	42	NS
Subtype[b]			
Tubular	12	29	
Papillary	48	57	
Br-alv[c]	6	0	0.015[e]
Differentiation[b]			
Well	31	19	
Moderate	24	43	
Poor	11	24	0.005
Scar grade[d]			
I, II	42	28	
III, IV	24	58	0.0003

[a]TNM staging according to Mountain's classification [20].
[b]Histological classification according to the Japanese Lung Cancer Society [21].
[c]Bronchioloalveolar type.
[d]Scar grade by Shimosato et al. [22].
[e]Br-alv versus others.
NS, not significant.

TABLE III. Association Between Cathepsin D in Tumor Cells and Cathepsin D in Stromal Cells

Cathepsin D-positive stromal cells	Cathepsin D in tumor cells: Negative (n = 80)	Positive: Granular type (n = 48)	Positive: Basal type (n = 24)
Few	46	15	5
Moderate to massive	34	33	19

$P = 0.0008$.

polarization might be strongly associated with the better differentiation, as described in non-small cell lung cancer by Fontanini et al. [18], and in breast cancer by Henry et al. [17]. Considering that cathepsin D is primarily a lysosomal enzyme to play a role in the intracellular degradation of exogenous and endogenous proteins [24], it is suggested that these original functions may be carried out in this type of lung adenocarcinoma as well as in the normal bronchial epithelium. By contrast, cathepsin D in basal type was mainly detected in the basal or infranuclear side of the cytoplasm, often in the basement membrane area of tumor cells. Cathepsin D itself is a proteolytic enzyme on proteoglycan substrates [2–4]; moreover, cathepsin D has been reported to activate cathepsin B [25,26], a cysteine proteinase, which is considered to raise the degree of malignancy in lung adenocarcinoma significantly as well as in several other malignant tumors by tumor invasion into the stroma [26–33]. Interestingly, the intracellular localization of cathepsin B has been found to change according to biological characteristics: in glioblastoma [32], cathepsin B was present throughout the cytoplasm in the more invasive tumor cells while it was detected in the perinuclear area

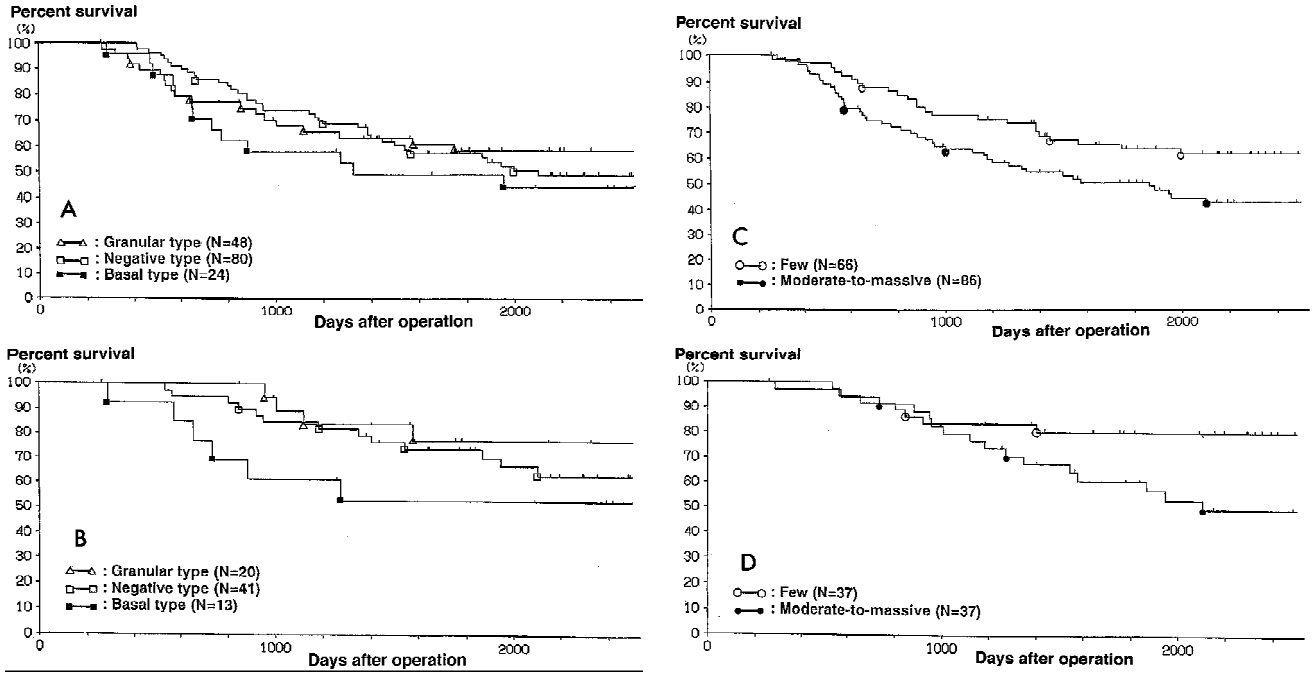

Fig. 2. Postoperative overall survival curves according to the pattern of cathepsin D expression in the patients with lung adenocarcinoma. **A,B:** Survival curves of the three groups according to the pattern of cathepsin D in tumor cells of all patients (A) or stage I patients (B). There was no significant difference among the three subgroups with all patients in the present series (A, $P = 0.306$). In stage I patients (B), no overall prognostic value was demonstrated ($P = 0.154$) among the three subgroups, but, in an individual analysis, patients with basal type expression showed marginally poor prognosis, compared to those in the other subgroups ($P = 0.071$). **C,D:** Survival curves of the two groups according to the extent of cathepsin D-positive stromal cells of all patients (C) or stage I patients (D). Patients with moderate to massive infiltration of cathepsin D-positive stromal cells showed significantly poorer prognosis than those with infiltration of few cathepsin D-positive stromal cells (C, $P = 0.014$), and the same result was observed among stage I patients (D, $P = 0.029$).

TABLE IV. Multivariate Analysis of Cox's Proportional Hazards Model for Postoperative Survival of Patients With Lung Adenocarcinoma

	Multivariate analysis			
Variable	Coeff	SE	χ^2	*P*
A. All patients (n = 152)				
p-stage	0.395	0.142	2.785	0.006
Tumor size	0.266	0.189	1.410	0.167
Differentiation	0.233	0.177	1.312	0.192
Scar grade	0.205	0.271	0.759	0.449
Cathepsin D in stromal cells	0.434	0.292	1.483	0.130
B. Stage I patients (n = 74)				
Tumor size	0.752	0.348	2.151	0.035
Scar grade	0.231	0.502	0.469	0.549
Cathepsin D in stromal cells	0.815	0.482	1.694	0.082

SE, standard error.

of the cytoplasm in the less invasive tumor cells, and in colorectal cancer [31], basal polarization of cathepsin B expression in tumor cells was noted, showing that this polarization may be reasonable for the invasive process. Therefore, cathepsin D expression in the basal type of lung adenocarcinoma may be directly, and/or indirectly via cathepsin B activation, associated with tumor invasion into stroma, and as a result, may slightly influence postoperative survival.

However, the problem of why cathepsin D expression in tumor cells biologically shows that two types of polarization pattern (i.e., granular type and basal type) has not been adequately explained. Cathepsin D is primarily produced as pro-cathepsin D (52 kD), and processed into the intermediate form (48 kD), and finally into the mature active form (34 kD). In neoplastic cells [34–37], this maturation of the pro-cathepsin D and its routing to lysosomes are well known to be altered, often resulting in its increased secretion into extracellular space. In fact, in breast cancer, the pro-form is not normally maturated, but increasingly secreted and activated extracellularly at the contact of basement membrane in an acidic microenvironment so that tumor cells may possess a higher potency of invasion into the stroma [34,35]. Therefore, it is speculated that the mechanism of these two types of cathepsin D polarization in tumor cells is associated with these alterations of pro-cathepsin D maturation, and especially in basal type, the mechanism may be similar to that in breast cancer. In this study, we used the polyclonal antibody that reacted with all these forms of cathepsin D [15,17], and cathepsin D expression in tumor cells was not qualitatively assessed in cathepsin D-positive pa-

tients including granular type and basal type, indicating the necessity of further analysis of the possible association between alterations of cathepsin D maturation and intracellular polarization of each form in lung adenocarcinoma cells.

Furthermore, cathepsin D expression in stromal cells within the tumor tissues, not tumors cells, is now considered to be a rather more important and useful prognostic factor [38,39]: in breast cancer, the larger the number of cathepsin D-positive stromal cells infiltrating within the tumor tissues, the worse is the prognosis [39], and similar results have been described in the other tumor tissues [40,41]. It was also experimentally suggested that cathepsin D-positive stromal cells might be more important for tumor aggressiveness [42]. In fact, the patients with moderate to massive infiltration of such cells in lung adenocarcinoma tissues showed poorer prognosis the those with less infiltration, and stromal cathepsin D status among stage I disease patients was a statistically marginal prognostic factor, indicating that cathepsin D status in stromal cells may be also a more useful indicator influencing prognosis than that in tumor cells in this disease.

Joensuu et al. [39] described that stromal cell cathepsin D expression in breast cancer was strongly associated with histologically less differentiation, and such a correlation was also found in lung adenocarcinoma ($P = 0.005$). The degree of cathepsin D-positive stromal cell infiltration within lung adenocarcinoma tisuses was significantly correlated with scar grade ($P = 0.0003$), and in the patients with basal type expression, cathepsin D-positive tumor cells were strongly distributed in the central scar area, admixed with the more massive cathepsin D-positive stromal cells, within the tumor tissues. Scar formation in lung adenocarcinoma is considered an important process for tumor growth, and it was described to be a prognostic factor [22]. On the basis of these observations, cathepsin D both in tumor cells and in stromal cells may play a key role in local invasion or metastatic potential of the tumor, possibly by interaction in the central scar, to have an impact on postoperative prognosis, although it may be statistically weak. In the bronchioloalveolar type of lung adenocarcinoma, which develops mainly along the alveolar wall and less into the stroma and uncommonly shows scar formation, it appears logical that cathepsin D expression in stromal cells was significantly uncommon.

In conclusion, in order to clarify clinicopathological and prognostic significance of cathepsin D expression in lung adenocarcinoma, it is important to analyze separately its status not only in cell type (tumor cells or stromal cells) but also its intracellular polarization in tumor cells (granular type or basal type). In lung adenocarcinoma patients undergoing potentially curative operation, cathepsin D expression status both in tumor cells and in stromal cells is a possible indicator of postoperative survival. However, only the latter status may be a statistically marginal independent prognostic factor among stage I patients.

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